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(54) Title: HERPES SIMPLEX VIRUS-1 DELETION VARIANTS AND VACCINES THEREOF

(57) Abstract

Novel Herpes simplex viruses and vaccines based on such novel HSV-1 strains are described. In particular, viruses ...ng a deletion in the terminal portion of R_L are provided. The virus can be further modified to express heterologous anti... ...d also engineered to overproduce HSV Light particles. This is achieved by incorporating a *ts* mutation into the UL26 ...

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Herpes Simplex virus-1 deletion variants and vaccines thereof

This invention relates to variants of herpes simplex virus type 1 (HSV-1) which lack neurovirulence. Such variants are of value in the preparation of live attenuated vaccines for the prevention of HSV infections in humans.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are important human pathogens which infect more than 80% of the general population and cause recurrent mucocutaneous lesions. Following replication HSV enters the peripheral nervous system where active replication is turned off by an unknown mechanism. Thereafter a latent infection in neurons is established which persists for the life of the host. HSV can reactivate from the latent state to produce infectious lesions. HSV is responsible for a broad spectrum of clinical diseases ranging from relatively benign cutaneous lesions to fatal viral encephalitis.

A considerable amount of research has already been devoted to elucidation of the genetic organisation of both HSV-1 and HSV-2. The HSV-1 genome is a linear double stranded DNA molecule of approximately 152 kilobase pairs consisting of two components L and S. Each component consists of unique sequences U_l and U_s, flanked by inverted repeats. The organisation of the HSV-2 genome is similar but not identical. For a detailed description of the genetic organisation of HSV-1 and HSV-2 (see McGeoch, 1987).

The identification of genes involved in viral pathogenicity and the elucidation of their precise functions is of fundamental importance to the understanding of the biology of herpes simplex virus (HSV). A number of variants of both HSV type 1 (strain 17) and HSV type 2 (strain HG52) with defined deletions in the unique and repeat sequences of both

the long and short regions of the viral genome have already been isolated and characterised (Brown et al 1984, Harland and Brown 1985, Brown and Harland 1987, MacLean and Brown, 1987a and b, Harland and Brown 1989). Little is known, however, about the molecular mechanisms which regulate the neurovirulence of HSV. It has been shown that a deletion variant of HSV-2 strain HG52, termed JH2604, is avirulent on intracerebral inoculation of mice (Taha et al, 1989a). JH2604 has ~ 1488 base pair deletion within both copies of the long repeat region of the genome [i.e. terminal long repeat (TR_L) and internal inverted long repeat (IR_L) regions].

An HSV-1 strain 17/HSV-2 strain HG52 recombinant (initially isolated in the Institute of Virology, Glasgow by Marsden et al, 1978), termed RE6, has also been reported to be avirulent in mice (Thompson et al 1989).

In HSV-1, inverted repeats of of the L component designated ab and b'a' are each approximately 9 Kbp whereas those of the S component c'a' and ca, are each approximately 6.5 Kbp. A sequence shared by the inverted repeats of the L and S components is designated the 'a' sequence. This sequence has been reported (Chou and Roizman 1986) to contain the promoter-regulatory sequence and the transcription initiation sites for a diploid gene located in the b sequence of the inverted repeats of the L component. Working with HSV strain F these authors reported that there was a transcribed open reading frame (ORF) between the 'a' sequence and an immediate early gene designated IE1. By the use of antipeptide sera they were able to show that the ORF specified a protein designated ICP 34.5 (Ackermann et al 1986). Recently Chou and Roizman (1990) have reported that their now predicted ORF is conserved in 2 other HSV-1 strains analysed but not in Glasgow strain HSV-1 (17) syn+. It has been suggested by Chou et al (1990) that the neurovirulence locus of HSV-1 comaps with, and requires the

expression of, ICP 34.5.

Surprisingly it has now been found that HSV-1 Glasgow strain 17 variants modified in the terminal portion of R_L lack neurovirulence.

Such variants are incapable of replicating in CNS neurons, but are able, in mice to elicit a good immunological and cell mediated response since they are capable of replication in the peripheral tissue. This ability emphasises the vaccine potential of these strains.

According to the present invention there is provided an HSV-1 strain the genome of which is modified in the terminal portion of R_L within Bam HI s (0-0.02 and 0.81-0.83 mu).

By Bam HI s it will be appreciated that what is meant is each copy of the approximately 3 Kb Bam HI s fragment of the HSV R_L region.

The term 'modified' is used herein to denote disruption of the Bam HI s fragment by deletion of one or more nucleotides, insertion of additional nucleotides or any other alteration of the nucleotide sequence such as rearrangement, or substitution.

The HSV-1 strain may be a spontaneously isolated deletion variant or may be a wild type strain into which the desired modification has been introduced.

Such modifications in the HSV strain may be made by genetic manipulation, for example by site-directed mutagenesis, or by excision of a portion of the genome with or without replacement with a pre-prepared DNA cassette incorporating

the required modification. Alternatively one may isolate naturally occurring HSV-1 variants, e.g. deletion variants.

Preferably the HSV-1 strain of the invention is a Glasgow strain 17 variant.

In one preferred aspect the HSV-1 strain is a strain in which at least 100 nucleotides in the Bam HI s' region between the Alu I site at 125074 np and 125972 np within the a sequence and its counterpart sequence in TR_L have been deleted.

More preferably 0.5 to 3 kb of the Bam HI s' region and its counterpart in TR_L is deleted, still more preferably about 0.7-2.5 kb is deleted.

In one specific example the HSV-1 variant is a strain designated 1714 which is a spontaneously occurring deletion variant of variant 1702 and lacks 759 bp within each copy of the Bam HI s fragment of the R_L region as described hereinbelow, in which the deletion associated with non-neurovirulence is located between nucleotide positions 125213 and 125972.

Such a deletion removes one complete copy of the 18bp DR_1 element of the 'a' sequence and terminates 1105bp upstream of the 5' end of the immediate early gene 1.

In another specific example the HSV-1 variant is a variant designated 1716 in which the 759 bp deletion in variant 1714 has been introduced into the wild type Glasgow strain 17^+.

In order to understand the invention more clearly reference may be made to Figure 1 hereinbelow in which:

(a) Shows the HSV-1 genome (with map units marked) in the prototype orientation; and

(b) Shows an expansion of BamHI k(s + g). The BamHI (B) and AluI (A) sites flanking the deletion in 1714/1716 are marked. All coordinates are based on the numbering of McGeoch et al (1988). Also indicated are the positions of the 5' end of IE1, the 'a' sequence, the DR_1/U_b boundary in the 'a' sequence, a 189bp conserved open reading frame between HSV-1 and HSV-2 (R_L ORF) and the end points of the 759bp deletion in 1714/1716. The deletion extends from the DR_1/U_b boundary to remove the 5' 107bp of the R_L ORF.

The present invention further provides a whole virus vaccine comprising an HSV-1 strain according to the invention wherein such vaccine comprises an immunoprotective and non-toxic amount of the strain of the invention. Such vaccine may comprise the strain alone or in conjunction with other antigens and/or adjuvants.

Due to their non-pathogenic nature, the viruses of the present invention are exceptional candidates for further modification. For example they may be further modified so as to carry heterologous antigens. The virus can be engineered so as to express antigens from HSV-2, such as HSV-2 gD. Such a virus, elicits both antibody and CTL responses to both type 1 and type 2 virus and, moreover, enhances the overall immune response. Similarly other antigens from the other pathogens may be presented by the viruses of the present invention. For example, gene products from HCMV, VZV, EBV, HHV6, HHV7, and HIV as well as other envelope viruses may be presented.

Moreover, the virus of the present invention may be modified by introducing a mutation, typically a temperature sensitive mutation into the gene UL26a which encodes the capsid

protein, P40 (Liu & Roizman 1991 a + b).

Such a mutation at non-permissive temperatures, (typically 38.5°C) results in the overproduction of light particles; that is virus particles lacking the nucleocapsid and nucleic acid, and hence infectivity. J. of Gen Virology (1991) 72 p661 Szilagyi and Cunningham.

Accordingly the present invention provides for light particles derived from the viruses described herein.

In a further embodiment, the present invention provides herpetic virus light particles carrying a heterologous antigen. For example in one embodiment of the present invention HSV-1 1716 has been modified to express HSV-2 gD, and also modified to contain a temperature sensitive mutation in UL26a gene at 38.5°C; this mutant over produces light particles containing HSV-2 gD. Other HSV-2 protein maybe incorporated into such a virus, in particular the HSV-2 gene products ICPO, ICP4 and Vmw 65 kD. Membrane proteins from other herpetic virus such as HCMV, VZV, EBV, HHV6, HHV7, and other enveloped virus such HIV-1 and HIV-2 maybe presented. For example gB from HCMV, gp120 from HIV-1 or HIV2 maybe incorporated into the virus Light particle. In theory any heterologous membrane protein which does not interfere with viral entry into the cell, can be carried by the light particles according to the invention.

Accordingly, the present invention provides a herpetic viral light particle carrying a heterologous antigen. In particular, the present invention provides a herpes simplex virus, preferably type 1, light particle carrying a heterologous antigen. An embodiment of this aspect of

invention is HSV-1 1716, $gD1^+$, gD_2^+, UL26a ts and light particles derived therefrom.

The Light particles of the present invention may be prepared by a modification of the method of Szilagyi & Cunningham (supra). Briefly cells are infected at 5 pfu/cell at the non-permissive temperature (npt) 38.5°C and the supernatant virus harvested at 30 hours post infection. This preparation is centrifuged on a preformed 5-15% Ficoll (made in Eagle's medium) gradient for 2 hours at 12 K. The Light particle band is removed with a 26 G needle and pelleted at 20 K overnight in normal cell growth medium (Eagles).

The light particles of the present invention are useful for vaccine purposes. Accordingly in a further aspect of the present invention there is provided a vaccine comprising a light particle from a herpetic virus carrying a heterologous antigen. In a further aspect there is provided a vaccine comprising an HSV-1 viral light particle derived from a virus comprising a modification in the terminal portion of R_L within BamH1 s (0-0.02 and 0.81-0.83 mu).

Alternatively, or in addition to the above mentioned modification(s), a virus of the present invention may be modified by introducing a mutation, typically a deletion, which renders the LAT promoter ineffective. Such a mutation adds a further level of safety, reducing both the frequency and rate of reactivation from latency.

Accordingly the present invention provides an HSV-1 virus modified in the terminal portion of R_L within BamHI s (0-0.02 and 0.81 - 0.83 mu) and also modified to render the LAT promoter ineffective. Such a modified virus may be further modified so as to produce heterologous antigens such

example HSV-2 gD, in the manner contemplated above. Moreover, additionally or alternatively to expressing a heterologous antigen, a temperature sensitive mutation maybe incorporated into the gene UL26a, so as to enable the overproduction of light particles and thus reduce the amount of potentially infective virus present. Such light particles may be separated from infective virus by Ficoll centrifugation of a viral particle. Normally, the ratio of heavy to Light particles in the Light particle band would be 1 : 10^3, however where a mutation in UL26a has been incorporated, the ratio of heavy to Light particles is typically in the order of 1 : 10^6.

The invention also provides a process for preparing a whole virus vaccine, which process comprises admixing the strain according to the invention with a suitable carrier or adjuvant.

For the preparation of a live attenuated vaccine, standard methodology may be used.

In a further aspect, the invention provides a method of treating HSV infection in humans, which method comprises administering to a human subject in need thereof an immunologically effective dose of the vaccine according to the invention.

The mode of administration of the vaccine of the invention may be any suitable route which delivers an immunoprotective amount of the strain or Light particle of the invention to the subject. However, the vaccine is preferably administered parenterally via the intramuscular or deep subcutaneous routes. Other modes of administration may also be employed, where desired, such as oral administration or via other parenteral routes, i.e., intradermally, intranasally, or intravenously.

The appropriate immunoprotective and non-toxic dose of such vaccine can be determined readily by those skilled in the art, i.e., the appropriate immunoprotective and non-toxic amount of the strain or Light particle of this invention contained in the vaccine of this invention may be in the range of the effective amounts of antigen in conventional whole virus vaccines. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, general health, sex, and diet of the patient; the time of administration; the route of administration; synergistic effects with any other drugs being administered; and the degree of protection being sought. Of course, the administration can be repeated at suitable intervals if necessary.

The following examples illustrate the invention.

Examples

METHODS

Cells

Baby hamster kidney clone 13 cells (BHK21/C13) (MacPherson and Stoker 1962) were propagated in Eagle's medium containing twice the normal concentration of vitamins and amino acids, 5% (V/V) tryptose phosphate broth and 10% (V/V) calf serum (ETC10).

Viruses

Virus stocks were grown and titrated in BHK21/C13 cells as previously described (Brown et al 1973). The parental HSV-1 strain was Glasgow strain 17 (Brown et al 1973). The variant 1702 devoid of the four normally occurring HSV-1 XbaI sites was the parental virus from which 1714 was isolated (MacLean and Brown 1987a).

Restriction enzyme analysis of virus genomes

Restriction enzyme analysis was carried out by a modification of the technique of Lonsdale (1979). BHK21/C13 cells were infected in the presence of $^{32}P_i$ in phosphate free Eagle's medium containing 1% (V/V) calf serum and incubated at 31°C for 48h. Viral DNA was extracted with SDS and phenol and ethanol precipitated. The DNA was treated with various restriction enzymes using the manufacturer's recommended conditions. Digests were analysed by electrophoresis on agarose gels of the appropriate concentrations (0.5-0.8%) in TBE buffer (89mM-Tris, 89mM

boric acid, 2mM sodium EDTA). Gels were air dried and exposed to Kodak XS1 film.

DNA-DNA Hybridisation

DNA fragments from restriction endonuclease digests were transferred from agarose gels to Hybond nylon membrane (Amersham) by the method of Southern (1975) and hybridised with random primed DNA prepared from Bam HI k (s + g) fragment cloned into PAT153. Hybridisation was performed at 65°C in a hybridisation buffer containing 7% SDS and 0.5M NaP, pH7 for 16h. No prehybridisation was performed. Filters were washed as described previously (Brown et al 1984).

Animal Inoculation

Three week old BALB/C mice (Bantin and Kingman) were inoculated intracranially with individual virus stocks. Mice were anaesthetised with ether and 0.025ml of the appropriate virus dilution in phosphate buffered saline (PBS) 5% calf serum was inoculated into the central region of the left cerebral hemisphere. Four mice were inoculated with each virus at doses between 10^1 and 10^7 pfu/animal. The virus stocks were always retitrated on the day of inoculation to determine the precise titre inoculated. Mice were observed daily after inoculation and the LD_{50} calculated according to the formula of Reed and Muench (1938) on the basis of death up to day 21. Brains were removed from animals which died post inoculation, homogenised, sonicated and the resulting suspension titrated on BHK21/C13 cells. Virus plaques were picked and their restriction enzyme profiles determined as described.

Virus growth properties in vitro

BHK21/C13 (2×10^6) cells were infected at a moi of 5 pfu/cell. Absorption was carried out for 45 min at 37°C and after two washes with phosphate buffered saline containing 5% CS and addition of a 2ml overlay of Eagle's medium containing 10% CS, incubation was continued at 37°C. Samples were harvested at 0,2,4,6,8,12,16 and 24h and virus released by sonication was titrated at 37°C.

Thymidine kinase assay

The method used was a modification of that of Jamieson and Subak-Sharpe (1974). BHK21/C13 cells were mock infected or infected with wild type or mutant virus at a moi of 5 pfu/cell. After absorption for 1h and incubation for 6h at 37°C, the cells were scraped into cold PBS and pelleted. The pellet was resuspended in lysis buffer (20mM Tris-HCl pH 7.5, 2mM $MgCl_2$, 10mM NaCl, 0.5% V/V Nonidet P40, 6.5mM 2 mercaptoethanol) maintained on ice for 5 min, mixed briefly and replaced on ice for a further 5 min. The samples were centrifuged and the supernatant retained. 5µl of extract was mixed with the reaction buffer in a total volume of 50µl (0.5M Na_2PO_4 pH6, 100mM $MgCl_2$, 2mM dTTP, 100mM ATP, 5µl aqueous Me^3H thymidine 1mCi/ml). After incubating for 1h the reaction was stopped by the addition of 10µl of 100mM EDTA and 1mM thymidine. The samples were heated for 3 min at 100°C and placed on ice for 3 min. After centrifugation, the supernatant was spotted onto DE81 discs which were washed 3 times (10 min each at 37°C) with 4mM ammonium formate pH 6.0 and 10µM thymidine. After a further 2 washes with ethanol, the discs were dried and radioactivity due to ^3H thymidine was determined.

Introduction of the deletion into wild type Glasgow strain 17

To introduce the 1714 deletion into wild type strain 17, the cloned novel BamHI k fragment of 1714 was linearised with BamHI and cotransfected at a 1,2,5,10 and 20 fold molar excess with intact DNA from 17^+. Resulting individual plaques were isolated and their DNA analysed by the method of Lonsdale (1979). Virus which appeared to have acquired the deletion was plaque purified a further 3 times before growing a virus stock.

Sequence Analysis

The novel BamHI k fragment of 1714 was cloned into the BamHI site of pGEM 3z using standard procedures (Maniatis 1982). Positive clones were identified by restriction enzyme analysis and confirmed by Southern blotting total HSV-1 DNA and using random primed DNA from the positive clones. Further restriction enzyme analysis confirmed that the deletion was approximately 800bp in size and was within a 2.8kb AluI fragment. This fragment was eluted from a gel, digested with SmaI and several small fragments were subcloned into M13mp8. Single stranded template DNA was prepared and sequenced using ^{35}S labelled dATP by the method of Sanger et al (1980). The sequencing products were run on a single concentration 6% acrylamide, 1 x TBE, 8.3M urea gel.

Latency studies

Three week old BALB/C mice (Bantin & Kingman) were inoculated in the right rear footpad as described previously (Clements & Subak-Sharpe 1983, 1988). At the time of inoculation the virus was titrated on BHK21/C13 cells to confirm the precise dose administered. For each virus a

series of 100 fold dilutions was inoculated and mice were examined and scored daily for clinical symptoms. Mice surviving 6 weeks were examined for the presence of latent virus. The mice were killed, dissected and the two lowest thoracic, six lumbar and the upper two sacral ganglia were removed from the inoculated side, placed in culture medium and screened for release of infectious virus every second day by transferring the culture medium to control BHK21/C13 cells. The inoculated BHK21/C13 cells were incubated at 37°C for 2 days before staining and examining for the presence of virus plaques or cpe.

Example 1

a) Isolation and genome analysis of the variant 1714

To study recombination in HSV we have constructed viruses devoid of certain restriction enzyme sites which are to be used as unselected markers (Brown et al 1984; Harland and Brown 1985; MacLean and Brown 1987c). The HSV-1 strain 17 mutant 1702 (MacLean and Brown 1987c) (devoid of the four HSV-1 XbaI sites and TK^-) was the parental virus used to remove various HindIII sites by site directed mutagenesis. DNA from the virus isolate H1 derived from 1702 but lacking the 0.91mu HindIII site was cotransfected with a mutagenised plasmid devoid of the 0.18mu HindIII site. A large number of resulting progeny plaques were picked and their DNA subjected to restriction enzyme analysis. In addition to successfully isolating a desired mutant in which the 0.18mu HindIII site had been lost, a virus (1714) with aberrant RE profiles unrelated to the loss of HindIII sites was detected.

On KpnI digestion of 1714 DNA KpnI r (2.4×10^6mw) was found to be missing and a novel band of about 1.9×10^6 was seen running between fragments t and u. KpnI r is the terminal

portion of R_L (0-0.025mu and 0.805-0.83mu) and forms the joint fragments a(r + j) and e(r + k). It can be seen that the 1714 e fragment is running marginally faster than its equivalent wild type fragment but no alteration in a which runs at the top of the gel can be seen. Similarly HpaI m (3.6×10^6 mw) was missing and a novel band of approx 3.1×10^6 mol wt was detectable running below n. HpaI m (0-0.036mu and 0.79-0.83mu) forms the joints a(m + c) and d(m + g) which can be seen in 1714 running marginally faster than 17^+. On BamHI digestion of 1714 DNA, BamHI s (1.95×10^6 mw) is missing and a new band appears to be running below u/v with a mol wt of about 1.45×10^6. The BamHI s containing joint k (s + g) is also not detectable but a novel band with a mol wt of 3.5×10^6 presumed to be the deleted joint is seen below l.

Taken together, the restriction enzyme profiles indicated that 1714 was deleted in both copies of the terminal portion of R_L between 0-0.095mu and 0.81-0.83mu. The size of the deletion was estimated to be between 600-800bp.

To substantiate the loss of sequences in both copies of R_L, Southern blot analysis of 1714 DNA was carried out. 17^+ and 1714 DNA were digested with BamHI and transferred to a nitrocellulose membrane. The BamHI k fragment (s + g) of 17^+ DNA was random primed and hybridised to the digested DNA. It was found that in the 17^+ track, the probe hybridised to k, g and s. In 1714 the probe failed to hybridise to k but hybridised to a novel k, to a novel k with additional 'a' sequences and to g. There was no hybridisation to s but to a novel s running below it. Incorporation of a size-ladder demonstrated the deletion to be about 800bp. This result unambiguously demonstrates that 1714 was deleted in both copies of R_L and that the deletion

was contained within BamHI s.

b) Sequence Analysis

The BamHI k joint fragment (s + q) of HSV-1 strain 17 is located between nucleotide positions (n.p.) 123459 and 129403 (McGeoch et al 1988). In 1714 the BamHI k fragment is deleted by about 800bp. This novel BamHI k of 1714 was cloned into the BamHI site of pGEM. Further restriction analysis indicated that the deletion lay within an AluI fragment (125074-127966 n.p.) which in the deletion variant 1714 was approximately 2.1kb in size compared to the wild type 2.9kb fragment. This AluI fragment from 1714 was eluted from an agarose gel, redigested with SmaI and the resulting subfragments were cloned into M13mp8. Dideoxysequencing of the SmaI fragments identified the deletion as being 759bp in length and located between nucleotide positions 125213 and 125972. From the remaining SmaI fragments sequenced, no other mutations were detected. The only precisely defined gene in R_L is IE1 whose 5' end in IR_L is located at 124108 n.p. i.e. 1105bp downstream of the deletion. The IR_L/IR_S 'a' sequence in HSV-1 strain 17 starts at nucleotide position 125954. In 1714 one complete 18bp DRI element (AGCCCGGGCCCCCCGCGG) of the 'a' sequence has been precisely removed.

Example 2

a) Neurovirulence of the deletion variant 1714 for Balb/c mice

We have previously shown that the deletion variant JH2604 of HSV-2 strain HG52 is non-neurovirulent for Balb/c mice with an LD_{50} value of $>10^7$ pfu/mouse compared to $<10^2$ pfu/mouse for the wild type virus. Sequence analysis of JH2604.

demonstrated that a 1488bp sequence within the terminal portion of the genome long repeat (between 0-0.02mu and 0.81-0.83mu) conferred neurovirulence on strain HG52.

As 1714 had a deletion in the equivalent parts of the HSV-1 genome, experiments to determine the neurovirulence of 1714 compared to its parent 1702 and to 17^+ by estimating their LD_{50} values in Balb/c mice were carried out. Twenty five µl aliquots of different doses of 17^+, 1702 and 1714 were inoculated into the left cerebral hemisphere of 3 week old Balb/c mice. Deaths from encephalitis were scored up to day 21 post inoculation and the results are shown in Table 1. The elite laboratory stock of 17^+ showed an LD50 value of $<10^{1.5}$ pfu/mouse. The mutant 1702, although tk negative (MacLean and Brown 1987a) gave a marginally higher LD_{50} value of 5×10^2 pfu/mouse. With 1714 no animals died with an inoculum of 10^6 pfu but 3/4 died with 10^7 pfu giving an LD_{50} value of 7×10^6 pfu/mouse. Thus the deletion variant 1714 was at least 2×10^4 fold less neurovirulent than the parental 1702 virus and at least 7×10^5 fold higher than the wild type 17^+. Single plaques were isolated from the brains of 1714 infected mice which had died and the DNA of the plaque isolates was digested with restriction enzymes. The RE profiles were identical to that of 1714, indicating no wild type contamination. The particle: pfu ratios of 72:1 for 17^+ and 58:1 for 1714 are comparable and fall within the normal range of values for HSV-1.

b) Growth of 1714 in vivo

The HSV-2 (HG52) variant JH2604 was shown to be avirulent; failed to replicate in mouse brain and produced no necrotising encephalitis (Taha et al 1990). To determine whether the absence of neurovirulence of 1714 was also due

to the failure of replication in mouse brain, samples of 17^+ (10^2 pfu), 1702 (10^2 pfu) and 1714 (10^5 pfu) were inoculated into the left cerebral hemisphere of 3 week old Balb/c mice. At various times post inoculation 2 mice (per virus) were killed, their brains removed and frozen at -70°C. The brain tissue was homogenised, the resulting suspension sonicated and the progeny virus assayed by plaque titration on BHK21/C13 cells at 37°C. The results showed that for the parental strain 17, there was exponential growth of virus between 12h post inoculation and day 6, reaching a final titre of 8×10^6 pfu/brain. Likewise with 1702 there was virus detectable 24h post inoculation and exponential growth reaching a titre of 8×10^4 pfu/brain by 6 days. In the 1714 infected animals which had received an input dose of 10^5 pfu, 2×10^3 pfu could be detected immediately post inoculation. No replication was detectable and the input virus declined until by 3 days post inoculation were was no assayable virus (<10 pfu).

c) Growth of 1714 in vitro

The variant 1714 grows to high titre (>10^9 pfu) by multicycle growth following low moi in BHK21/C13 cells. The stock gives equivalent titres when assayed at 31°C, 37°C, and 38.5°C. To determine its growth pattern, single cycle growth experiments were carried out in BHK21/C13 cells at 37°C. The results showed that 17^+ and the 1702 and 1714 variants grew equally well giving equivalent final yields. The normal single cycle growth pattern of 1714 indicates no impairment at any stage in its replicative cycle in BHK21/C13 cells.

The determine whether the virus was host restricted, 24h yield experiments were carried out in a range of cell lines infected at a moi of 5 pfu/cell. The cell lines used were BHK/C13 (hamster), BSC1 (monkey), Vero (monkey), MDCK (dog),

HFL (human) and 3T6 (mouse). The 24h yields in BHK21/C13 cells titrated at 37°C are shown in Table 2 as are the ratios of the yields of virus grown in a particular cell line compared to the yield in BHK21/C13 cells. It can be seen that 17^+, 1702 and 1714 essentially behave in a similar fashion; they grow equally well in BHK21/C13, 3T6 and MDCK cells, better in Vero cells and less well in HFL and in BSCI cells. Note that there was no replication defect in the mouse 3T6 cells demonstrating that the lack of growth in vivo was not species specific.

Example 3

Latency Studies

Three week old Balb/c mice were inoculated in the right rear footpad with serial 10 fold dilutions of 17^+, 1702 and 1714 (4 mice/dose) and were monitored daily for two weeks for signs of illness or death. At 6 weeks post inoculation, surviving mice were dissected as outlined in METHODS (above) and ganglia were separately transferred to microtitre wells containing culture medium. Screening for the presence of infectious virus was carried out every second day post explantation, by transferring an aliquot of culture medium to control BHK21/C13 cells. The cells were then incubated at 37°C for 2 days before staining and examining for the presence of virus plaques or cpe. The results in Table 3 show that at doses of 10^4 and 10^5 pfu of 17^+, 20% of explanted ganglia reactivated. However, soon after inoculation one of the 10^4 pfu and 3 of the 10^5 pfu infected animals developed hind limb paralysis and had to be killed. Animals were not inoculated with 10^6 pfu of 17^+ as they would all have been expected to die. With 1702 infected animals, 5% of ganglia at dose of 10^4 and 10^5 pfu reactivated and 17.5% reactivated at the 10^6 pfu dose. This

was clearly less efficient than 17^+ possibly due to the tk negative phenotype of this variant. With 1714 inoculated animals, no qanglia reactivated from 10^4 pfu infected animals, only 1/40 (2.5%) reactivated from the 10^5 pfu infected mice and 2/40 (5%) from the 10^6 pfu infected animals. Virus first reactivated at day 6 post explantation and there was no significant difference in the timing of reactivation between the 3 viruses. Virus reactivation was confined to the lumbar ganglia in the 3 groups of mice.

Taking into account the tk negative phenotype, the variant 1714, although capable of latency, was much less efficient than 1702 in establishing the latent state and/or reactivating from it following explantation.

Example 4

Introduction of the 1714 deletion into the 17^+ wild type genome

As the 1714 deletion was not in a wild type background ie the four Xbal sites at 0.07, 0.29, 0.45 and 0.63mu were deleted in the genome and the virus was tk negative, it was conceivable that its avirulent phenotype was at least in part due to these other mutations. It seemed very unlikely as the parent strain 1702, which contains the same Xbal negative and tk negative mutations had a virulence phenotype essentially equivalent to 17^+. Nonetheless we decided to introduce the deletion in 1714 into an otherwise totally wild type genome. 17^+ DNA was co-transfected with a 10-fold excess of plasmid cloned BamHI k of 1714. Resultant single progeny plaques were isolated and their DNA profiles analysed by the method of Lonsdale (1979). A virus with a 1714 BamHI profile designated 1716 was isolated, plaque purified a further 3 times and a virus stock grown. To

confirm that 1716 has retained its wild type background in respect of XbaI sites and tk activity, the DNA of 1716 was digested with XbaI and a tk assay performed. It was found that 1716 had a normal wild type XbaI profile retaining the four sites in U_L whereas 1714 and 1702 fail to digest with XbaI. The results of the tk assay for 1716 compared to 17^+, 1702 and 1714 are given in Table 4 and demonstrate that 1716 is as efficient as 17^+ in synthesising tk. The neurovirulence phenotype of 1716 was tested by IC inoculation of Balb/c mice. Its LD_{50} value compared to 17^+ and 1714 is shown in Table 5. It can be seen that it is non-neurovirulent with an LD_{50} value of 7×10^6 pfu/mouse while in this experiment 17^+ had an LD_{50} value of <10 pfu/mouse confirming that the sequences deleted in 1716 confer neurovirulence on strain 17.

Results of a single cycle growth experiment with 1716 showed that 1716 grows as efficiently as wild type 17^+ virus.

Table 1

Intracerebral (IC) inoculum/mouse (pfu)

Virus	10^2	10^3	10^4	10^5	10^6	10^7	LD_{50} (pfu/mouse)
17+	4/4*	4/4	ND	ND	ND	ND	$<10^{1.5}$
1702	2/4	3/4	4/4	4/4	ND	ND	5×10^2
1714	ND	0/4	0/4	0/4	0/4	3/4	7×10^6

* Number dead/Number inoculated

ND = Not done

Table 2

Cell type	BHK21/C13 standard	Vero	BSC1	3T6	MDCK	HFL
Virus						
17+	7.6×10^6*	1.83+	0.43	0.98	0.68	0.65
1702	5.2×10^6	2.8	0.48	1.31	1.11	0.29
1714	5.6×10^6	1.34	0.39	0.97	1.07	0.42

* Virus yield over 24h at 37°C expressed as pfu/5×10^5 cells

+ Ratio of yield of virus in the particular cell type compared to the yield in BHK21/C13 cells.

Table 3

Inoculating dose Virus	10^4 pfu/mouse	10^5	10^6
17^+	6/30* (20^+)	2/10 (20)$^{++}$	ND
1702	2/40 (5)	2/40 (5)	7/40 (17.5)
1714	0/40 (0)	1/40 (2.5)	2/40 (5)

* No. of ganglia reactivating/No of ganglia explanted

$^+$ % of reactivating ganglia

$^{++}$ Four animals were infected/dose and 10 ganglia explanted from each.
With 17^+ infected animals, 1 animal at 10^4 pfu and 3 at 10^5 pfu dose developed paralysis soon after infection and had to be killed.

Table 4

tk assays on Glasgow strain 17 and the variants 1714 and 1716

	Radioactivity cpm/µg protein
Mock infected	11267
Glasgow strain 17	143894
1714	8399
1716	131987

Table 5

Intracerebral (IC) inoculum/mouse (pfu)

Virus	10^1	10^2	10^3	10^4	10^5	10^6	10^7	LD_{50} (pfu/mouse)
17^+	4/4*	4/4	ND	ND	ND	ND	ND	<10
1714	ND	ND	ND	ND	0/4	0/4	4/4	5×10^6
1716	ND	ND	ND	0/4	0/4	0/4	3/4	7×10^6

* No. dead/No. inoculated

ND = Not done

Example 5

Construction of HSV-1 1716 $gD1^+$, $gD2^+$.

A recombinant plasmid containing the Hind III l fragment of HSV 2 strain HG52 (McGeoch **et al** 1987a) was digested with restriction endonucleases Bst EII and Dra I and a 3Kb fragment from the Dra I site at np 5893 to the BSt EII site at np 8893 purified. This fragment contains the promoters, open reading frames and poly A signal of the 3' coterminal genes UL6 (gD-2) and US7 (gI-2). The 5' overhang of the Bst EII site was blunt ended using klenow polymerase. This gD-2 containing fragment was inserted into a Bam HI/ECoR1 91610/96751 np fragment of HSV1 containing UL43 (McGeoch et al 1988) a non-essential integral membrane protein (Maclean C et al 1991). The site of insertion was a unique Nsi I site np 94911 at the 5' end of UL43. The 5' overhang of the Nsi I site was blunt ended using klenow polymerase. All cloning techniques are as described by Maniatis et al 1982.

The recombinant UL43 gD2 HSV1 fragment was cotransfected with intact HSV1 1716 variant DNA and recombinant genomes isolated as described (Example 4 and Maclean et al 1991). A HSV recombinant containing gD2 was isolated. This virus $gD1^+$ gD^{2+}, ICP34.5- is known as 1761.

Example 6

a) **Construction of HSV-1 1716 UL26 ts.**

The Cloned ECORI f fragment of ts 1201 (Preston et al 1983) contains the UL 26 gene with a ts point mutation. This was recombined into 1716 to generate HSV - 1716 UL26 ts as previously described in example 4.

Construction of HSV-1 1716 $gD1^+$ gD_2^+ UL26 ts.

b) HSV-1 1716 UL26 ts and HSV-1 1716 $gD1^+$ gD_2^+ from the above examples is recombined using standard methodology (Brown et al 1973) to give an HSV-1 1716 $gD1^+$ $gD,2^+$, UL26 ts virus.

Example 7

Construction of 1716 $gD1^+$, $gD2^+$ LAT P^- and 1716 $gD1^+$, $gD2^+$ UL26 ts LAT P^-.

A fragment (Steiner et al 1989, JuneJo et al 1991) isolated from HSV-1 1704 carries a 942 bp deletion in both copies of the LAT promoter. This fragment is cotransfected with 1716 $gD1^+$ $gD2^+$ and 1716 $gD1^+$, $gD2^+$, UL26, DNA and single plaques analysed, to give 1716 $gD1^+$ $gD2^+$ LAT P^- and 1716 $gD1^+$ $gD2^+$, UL26 ts, LAT P^-

HSV-1 strains 1714 and HSV strains 1716 have been deposited at the European Collection of Animal Cell Cultures, Vaccine Research and Production Laboratories, Public Health Laboratory Services at Porton Down, Salisbury Wiltshire SP4,0J9, UK on 28th January 1992 and given the accession Numbers V92012802 and V92012803 respectively.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 26, line 20 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution

European Collection of Animal Cell Cultures, Vaccine Reseach and Production Laboratories

Address of depositary institution (including postal code and country)

Public Health Laboratory Services, Porton Down, Salisbury, Wiltshire SP4 0J9

Date of deposit	Accession Number
28 January 1992	V92012802

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

31 03 92

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☐ The date of receipt (from the applicant) by the International Bureau is

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(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 26, line 20 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet [X]

Name of depositary institution

European Collection of Animal Cell Cultures,
Vaccine Research and Production Laboratories

Address of depositary institution (including postal code and country)

Public Health Laboratory Services, Porton Down
Salisbury, Wiltshire SP4 0J9

Date of deposit	Accession Number
28 January 1992	V92012803

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

In respect of those designations in which a European Patent is sought, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [ ] This sheet was received with the international application when filed (to be checked by the receiving Office)

31-05-92

(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau was

(Authorized Officer)

Form PCT RO/134 (January 1981)

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Claims

1. An HSV-1 strain, the genome of which is modified in the terminal portion of R_L within Bam H1 s (0-0.02 and 0.81-0.83 mu).

2. An HSV-1 strain as claimed in claim 1 wherein the genome is modified by a deletion.

3. An isolated naturally occurring HSV-1 deletion variant wherein the genome is modified in the terminal portion of R_L within the Bam H1 s (0-0.02 and 0.81-0.83 mu).

4. An HSV-1 strain as claimed in claim 1 or 2 in which at least 100 nucleotides in the Bam HI s region between Alu I site at 125074 np and 125972 np and its counterpart in TR_L have been deleted.

5. An HSV-1 strain as claimed in claim 1, 2 or 4 in which at least 0.5 to 3 Kb of the Bam HI s region and it's counterpart in TR_L is deleted.

6. HSV-1 strain as claimed in claim 1, 2 or 4 in which at least 0.7-2.5 Kb of the Bam HI s region and it's counterpart in TR_L is deleted.

7. HSV-1 1714.

8. HSV-1 1716.

9. An HSV-1 strain as claimed in any of claims 1 to 8 in which the strain has been further modified to carry a heterologous gene.

10. An HSV-1 strain as claimed in claim 9 wherein the heterologous gene is selected from the group HSV-2 gD, HCMVgB, HSV-2 ICP_0, ICP_4, VMW65, HIV-1 gp120 and HIV-2

gp120.

11. An HSV-1 strain as claimed in any of claims 1 to 9 in which the strain has been further modified by incorporating a temperature sensitive mutation into the UL26 gene.

12. An isolated light particle preparation, derived from a herpetic virus, carrying a heterologous antigen.

13. A Light particle preparation as claimed in claim 12 wherein the heterologous antigen is selected from HSV gD, HCMV gB, HSV-2, ICP_0, ICP_4, VMW65, HIV-1 gp120 and HIV-2 gp120

14. An HSV-1 strain as claimed in any of claims 1 to 11, further modified by incorporating a mutation rendering the LAT promoter ineffective.

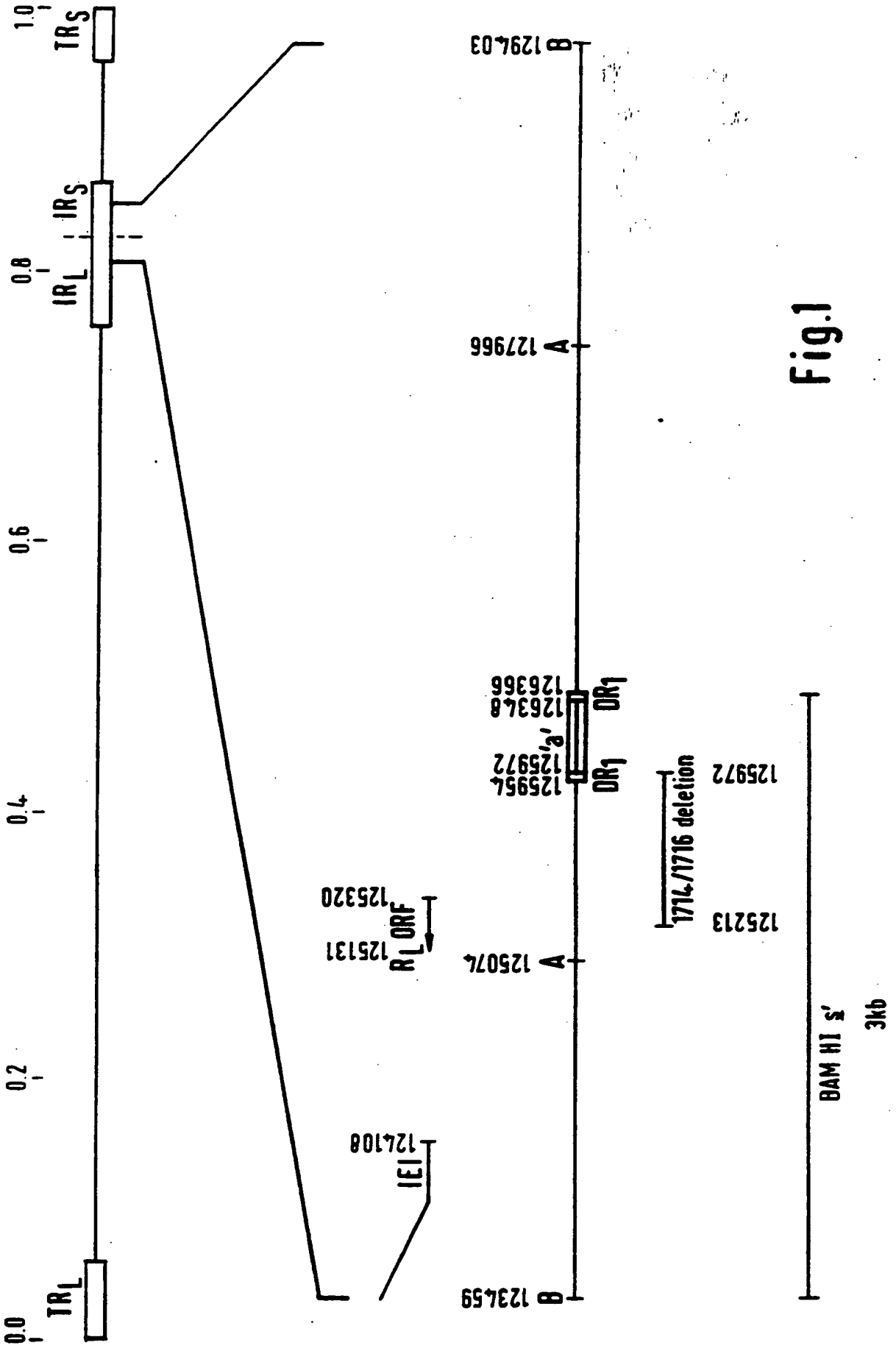
15. A light particle derived from a strain of claim 14.

16. A vaccine comprising an HSV-1 strain as claimed in any of claims 1 to 11, or 14, in admixture with a pharmaceutically acceptable excipient.

17. A vaccine comprising a light particle as claimed in any of claims 12, 13 or 15 in admixture with a pharmaceutically acceptable excipient.

18. A method of treating a patient susceptible to HSV infections comprising administering to a human subject in need thereof an immunologically effective dose of the vaccine of claim 16 or 17.

19. A process for the preparation of an HSV-1 strain as claimed in claim 1, comprising modifying the genome of said strain, in the terminal portion of R_L within Bam HI s (0-0.02 and 0.81 - 0.83 mu).


1.0
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126348
DR1
'a'
125972
125954
DR1
1714/1716 deletion
125972
125213
125320
RL ORF
125131
A 125074
124108
IE1
B 123459
BAM HI s'
3kb

Fig.1

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00179

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 7/04 A 61 K 39/245

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X,P	Journal of General Virology, vol. 72, part 3, March 1991, (London, GB), A.R. MacLEAN et al.: "Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence", pages 631-639, see the whole article	1-8
Y	Journal of General Virology, vol. 70, 1989, M.Y. TAHA et al.: "A variant of herpes simplex virus type 2 strain HG52 with a 1.5 kb deletion in RL between 0 to 0.02 and 0.81 to 0.83 map units is non-neurovirulent for mice", pages 705-716, see the whole article, specially pages 706-707: "Methods"; pages 713-715: "Discussion"	1-6,9-10,16-19

-/-

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14-04-1992	23. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0243155 (INSTITUT MERIEUX) 28 October 1987, see the whole document, specially page 8, lines 1-8; page 13, lines 23-32; page 14, lines 1-11; pages 33-36, claims	1-6,9-10,16-19
X	EP,A,0175261 (CHIRON CORP) 26 March 1986, see the whole document	12-13
A	Journal of General Virology, vol. 68, 1987, A.R. MacLEAN et al.: "Deletion and duplication variants around the long repeats of herpes simplex virus type 1 strain 17", pages 3019-3031, see the whole article	1-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 4-14 because they relate to subject matter not required to be searched by this Authority, namely:

REMARK: ALTHOUGH CLAIM 18 IS DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOSITION.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple Inventions in this International applica... follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200179 SA 55947

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/05/92 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0243155	28-10-87	US-A- 4859587	22-08-89
		AU-B- 585126	08-06-89
		AU-A- 6861487	29-10-87
		JP-A- 62257385	09-11-87
EP-A- 0175261	26-03-86	CA-A- 1263618	05-12-89
		DE-A- 3584866	23-01-92
		JP-A- 61129135	17-06-86
		US-A- 4722840	02-02-88

For more details about this annex : see fficial Journal of the European Patent Office, N . 12/82